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Antigen binding of antiganglioside antibodies in vitro is strongly influenced by the ganglioside composition of the sample

Herbert Hildebrandt*

Institut für Zoologie (220), Universität Hohenheim, Garbenstraße 30, 70593 Stuttgart, Germany

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Abstract The ability of antiganglioside antibodies to detect their respective antigens in various environments was studied. In contrast to sensitive detection of pure ganglioside standards by ELISA, antibody binding to mixtures of gangliosides was drastically reduced. This loss of sensitivity also occurred with immunostaining of gangliosides absorbed to silica gel. Moreover, absorption of antibodies to antigen-containing lipid vesicles failed, if the vesicles were prepared together with other gangliosides. These data indicate that antigen accessibility is strongly influenced by surrounding gangliosides. This should be considered whenever gangliosides are traced by antibodies. The ELISA procedure appears useful to test for such properties.

Key words: Glycosphingolipid; Sialic acid; Antigen accessibility; Membrane glycolipid; Tumor marker; Cell surface

1. Introduction

During the last decades an increasing number of specific, monoclonal antiganglioside antibodies have been generated. They have been used to analyse the occurrence and biological functions of gangliosides(1) [2–7] and serve as powerful tools in the diagnosis and therapy of tumours of neuroectodermal origin such as melanoma or neuroblastoma [8–11]. Besides binding to gangliosides in situ, antiganglioside antibodies were applied to trace gangliosides in vitro, mainly using ELISA or immuno-thin layer chromatography (immuno-TLC) [6,7,12–20]. Both techniques allow detection in the picomolar range. Particularly with the ELISA approach ganglioside antigens could be determined in a sensitive, dose-dependent manner [14–18]. But up to now ELISA has merely been applied to purified ganglioside standards in order to assess specificity and dilution of antibodies. Only the well known affinity of cholera toxin towards GM1 has been used in an ELISA-like assay to estimate the amount of GM1 in ganglioside samples isolated from neuro-2a and PC 12 cells [21]. On the other hand, the use of immuno-TLC is restricted since quantitative evaluation is difficult [13] and TLC separation of gangliosides extracted from cells or tissues still requires tedious purification procedures [18–20,22]. Nevertheless, the specific antiganglioside antibodies seem to provide a convenient tool to detect small amounts of ganglioside antigen in a sample. Consequently, the ability of such antibodies to bind their respective antigens in different environments was tested. This study demonstrates a dramatic loss of the antibodies' sensitivity if ganglioside species other than the respective antigens are present in a sample.

*Corresponding author. Fax: (49) (711) 459 3450.

Abbreviations: BSA, bovine serum albumin; CT-B, cholera toxin B subunit; DOPC, dioleoylphosphatidylcholine; ELISA, enzyme-linked immunosorbent assay; G_{mix}, standard ganglioside mixture as described in section 2.3; HRP, horseradish peroxidase; HSA, human serum albumin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TLC, thin layer chromatography

(1) Gangliosides are named according to the nomenclature of Svennerholm [1].

2. Materials and methods

2.1. Materials

HRP-labelled cholera toxin B subunit (CT-B-HRP) was from RBI (Natick, MA, USA), dioleoylphosphatidylcholine (DOPC) from Avanti Biochemicals (Alabaster, AL, USA) and cholesterol from Sigma (Deisenhofen, Germany). TLC plates (Silica gel 60) were obtained from Merck (Darmstadt, Germany), Immulon-1 microtiter plates from Dynatech (Denkendorf, Germany).

2.2. Antibodies

IgM mAb GMB16, GMR17 and GMR5 containing supernatants of hybridoma cells were kindly provided by Dr T. Tai, Tokyo. Affinity purified mAb R24, IgG3, was a gift from Dr K. Lloyd, New York. As determined by ELISA and immunostaining on TLC, GMB16 and GMR5 were reported to be specific for GM1 and GT1b respectively, whereas GMR17 strongly reacts with GD1a, but showed some cross-reactivity with GT1b and GM1b [15,16]. R24 initially was described to be specific for GD3 [23]. In a later study, however, some cross-reactivity to disialylparagloboside was reported [14]. Phosphatase labelled secondary antibody (goat) against mouse IgG (whole molecule) was purchased from Sigma (Deisenhofen, Germany).

2.3. Standard gangliosides

Gangliosides GD3, GM1, GD1a, GT1b and a standard mixture G_{mix} consisting of GM1 (22%), GD1a (40%), GD1b (18%), GT1b (20%) with traces of GQ1b were purchased from Dr. Pallmann GmbH (Munich, Germany). Purities of the standard gangliosides were 99.8% (GD1a, GD3), 99.2% (GM1), and 98% (GD1a) as analysed by two-dimensional TLC, staining with resorcinol or H_2SO_4 reagent and densitometric scanning (Pallmann, personal communication). All standards were stored as 1 mM aqueous stock solutions.

2.4. Ganglioside extraction

A crude chloroform/methanol extract containing gangliosides and other polar lipids was prepared from homogenised rat brain. An aliquot, corresponding to 15 mg of tissue (wet weight), was lyophilised, sonicated twice with cold acetone (500 μl, −20°C) and centrifuged at 10 000 × *g* for 10 min. The supernatant, containing non-polar lipids (but no detectable sialic acid), was discarded. To obtain all remaining lipids, the residue was dried and extracted twice with 500 μl chloroform/methanol 1:1 according to Li and Ladisch [24,25]. After evaporation of the solvent the combined chloroform/methanol extracts were redissolved in water. Aliquots were taken for determination of lipid-bound sialic acid using the method of Svennerholm [26] as modified by Miettinen and Takki-Luukainen [27].

2.5. Immunostaining on TLC plates

Using a syringe, G_{mix} was repeatedly applied as a fine line to the

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starting site of the TLC lane, separated by TLC using the solvent system chloroform/methanol/12 mM $MgCl_2$/37% NH_3 60:40:9:0.1 and stained either by resorcinol spray [26] or by immuno-TLC as described elsewhere [13,28]. If gangliosides absorbed to a TLC plate were immunostained without previous separation, the respective amount of ganglioside was applied by drops.

2.6. ELISA and GM1 assay with CT-B-HRP

ELISA was performed according to Kotani et al. [16] and Ozawa et al. [15], but alkaline phosphatase was used as a more convenient detection system and 2% BSA was used instead of HSA. To obtain ganglioside mixtures at the desired concentration, samples or standards were first combined and diluted with water and subsequently mixed with ethanol to give a 70% ethanol solution. Per well, 10 μl of this solution was added to 50 μl of ethanol, mixed and evaporated to complete dryness. After washing (PBS) and blocking (2% BSA in PBS, 2 h) samples were incubated for 3 h (at room temperature) or overnight (at 4°C) with 100 μl of primary antibody in BSA-PBS. If not stated otherwise, dilutions of 1:500 (mAb R24) or 1:20 (all other mAbs) were used. After washing, alkaline phosphatase labelled secondary antibody (1:1000 in blocking buffer) was applied for 2 h followed by another washing step and staining with *p*-nitrophenylphosphate in Tris-HCl, pH 8.8. Absorbance at 405 nm was determined by a SLT ELISA plate reader.

Assays for GM1 using CT-B-HRP were performed according to Wu and Ledeen [21], but gangliosides were coated as described above. 20 ng of CT-B-HRP was added per well and incubated for 1 h at room temperature. After washing with PBS and 0.1% Tween 20/PBS, CT-B-HRP was detected by the peroxidase substrate tetramethylbenzidine (TMB, 0.4 mM in 1% H_2O_2/PBS). Reaction was stopped with 10 μl 1 M H_2SO_4 and quantified at 450 nm.

2.7. Lipid vesicles

Mixed DOPC-cholesterol-ganglioside vesicles were prepared from a chloroform/methanol (1:1, v/v) solution containing 500 nmol of DOPC, 500 nmol of cholesterol and 20 nmol of ganglioside. After evaporation of the solvent under a gentle flow of N_2 the lipids were redissolved in 500 μl *n*-hexane, shaken vigorously (overnight at 4°C) and dried again. After addition of 2 ml PBS (pH 7.4) the dispersion was sonicated intensely, to obtain mixed vesicles (see [29] and references therein).

3. Results

ELISA was performed with different antibodies. Ganglioside antigen was coated either as pure standard or as part of a mixture of standard gangliosides. As shown in Fig. 1, detecting different concentrations of pure standard ganglioside by the appropriate antibody resulted in calibration curves that were linear in the range of 10–100 pmol of ganglioside. However, if the microtitre plate was coated with the same amount of the particular ganglioside antigen but mixed with other gangliosides (G_{mix}), a drastic decrease in sensitivity occurred. In contrast, using CT-B-HRP for specific detection of GM1 [21], sensitivity decreased only slightly, if the relative amount of GM1 was reduced to 22% of total ganglioside. To further assess this inhibition of antibody binding due to the presence of gangliosides other than the respective antigen, the relative amount of antigen was varied in two ways. The relative amount of e.g. GM1 was varied (i) by mixing different amounts of GM1 and GD1a to a total of 100 pmol of ganglioside or (ii) by adding different amounts of GD1a to a fixed amount of 100 pmol GM1. Fig. 2 demonstrates a non-linear increase of antibody binding with increasing relative amounts of the ganglioside antigen. In both cases, i.e. with the fixed amount of 100 pmol GM1 as well as with the fixed amount of 100 pmol total ganglioside, the binding increased strongly with decreasing amounts of GD1a added (Fig. 2a). The same results were obtained with addition of GM1 to GD1a

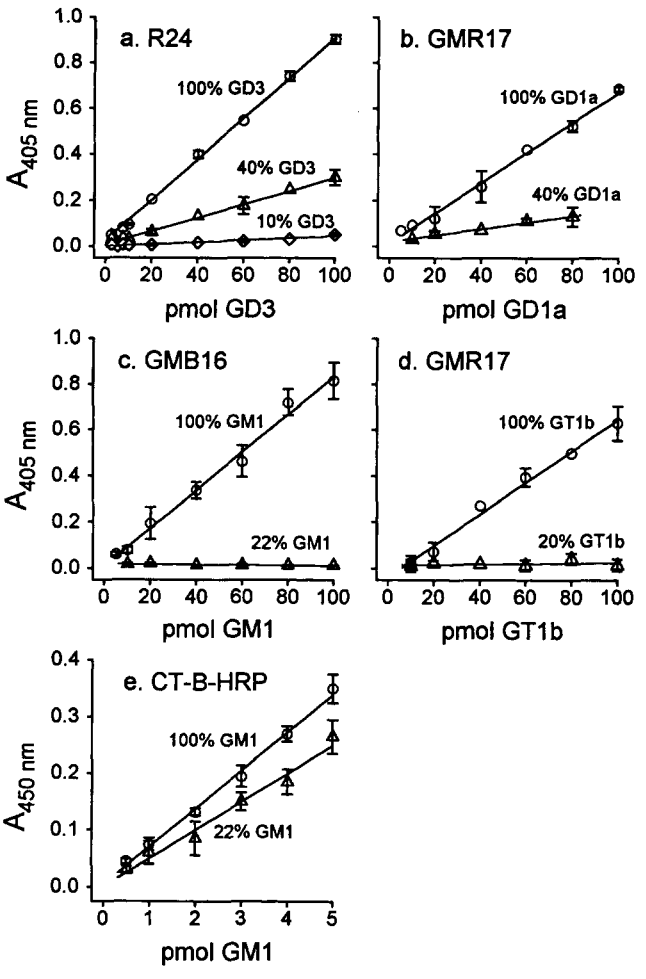

Fig. 1. Reactivity of four antiganglioside antibodies with different amounts of their respective ganglioside antigens determined by ELISA (a–d) compared to detection of GM1 by CT-B-HRP (e). Gangliosides were either coated as pure standards (100%, ○, a–e) or at the percentages indicated as part of G_{mix} (b–e) or as addition of GD3 to G_{mix} (a). Values are means of 2–5 determinations (±S.D. if $n > 2$).

(Fig. 2b). Thus, antibody binding seems to depend mostly on the proportion of antigen relative to total ganglioside contents and not on the absolute amount of ganglioside antigen.

Therefore, to quantify the absolute amount of a ganglioside antigen by ELISA, standards and samples should have the same relative ganglioside composition, a prerequisite that cannot be accomplished for unknown samples. Trying to overcome this difficulty, increasing amounts of standard ganglioside antigen were added to aliquots of crude rat brain extract and the resulting calibration curve was extrapolated to estimate the amount of antigen that had originally been present in this sample (Fig. 3). Since the relative amount of antigen increased with addition of external antigen, a non-linear rise in antibody sensitivity was expected (see Fig. 2), so that a linear regression tends to underestimate the amount of endogenous ganglioside. Nevertheless, the amount of 6.5 pmol GD3 estimated by the linear extrapolation procedure (equal to 1.2% of total lipid-bound sialic acid) closely matches published values of ganglioside composition determined for rat brain (1.1% GD3 [22]). In contrast, the estimated amount of GD1a (20 pmol, 27% of total lipid-bound sialic acid) is some-

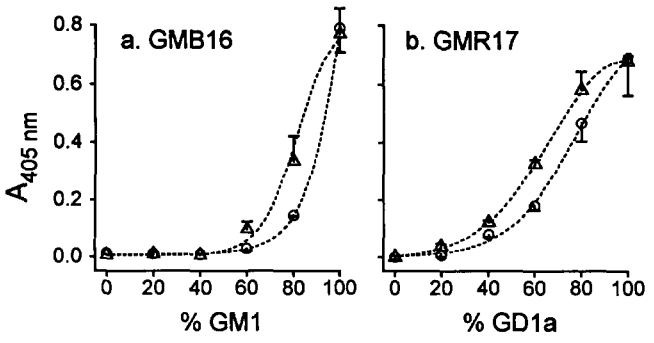

Fig. 2. Reactivity of mAb GMB16 (a) and mAb GMR17 (b) with different relative amounts of GM1 or GD1a determined by ELISA. Values are means (±S.D.) of 3 determinations each. (a) GM1 and GD1a were either mixed to a final amount of 100 pmol ganglioside (○) or increasing amounts of GD1a were added to a fixed amount of 100 pmol GM1 (△, 1 nmol of GD1a was coated for the value at 0% GM1). (b) Same as (a) but GD1a was used instead of GM1 and vice versa.

what lower than values reported in the literature (30–35% GD1a [22,30]). Although GMR17 shows some cross-reactivity with GT1b and GM1b [16], controls with addition of 40 pmol of GT1b revealed no detectable increase of antibody binding (data not shown). Thus, GT1b, a major ganglioside of the rat brain, did not interfere with the present assay.

In order to elucidate the influence of ganglioside composition on antibody binding to samples absorbed to surfaces other than polystyrene, pure ganglioside antigen or G_{mix} were absorbed to TLC plates and immunostained without separation (Fig. 4a). In accordance to the results obtained by ELISA, clearly detectable antibody binding occurred only if the antibody was exposed to a surface impregnated with pure antigen. In a control experiment it was demonstrated that after TLC separation the antibodies were able to detect the ganglioside fractions contained in G_{mix} (Fig. 4b).

The ability of antibodies to bind to gangliosides incorporated into mixed phospholipid-cholesterol vesicles was analysed in a pre-absorption experiment. Antibodies were incubated with vesicle preparations containing either a mixture of gangliosides or the respective antigen as the only ganglioside. Thereafter, unbound antibody in the antibody/vesicle solution

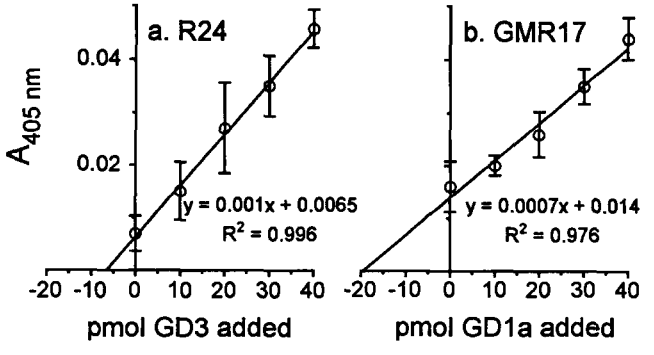

Fig. 3. Reactivity of mAb R24 (a) and mAb GMR17 (b) in ELISA with crude rat brain extract and different amounts of GD3 or GD1a added as indicated. Total lipid-bound sialic acid of brain extracts was 2.5 nmol per mg wet weight. After addition of standard ganglioside, brain extract equivalent to 1.1 (a) or 0.15 nmol sialic acid (b) was coated per well. Values are means (±S.D.) of 3 or 4 determinations each. Linear regression analysis was applied and curves were extrapolated towards y=0.

was assessed by means of ELISA. Fig. 5 demonstrates that antibody binding to its specific antigen in ELISA was inhibited, if the antibody was preabsorbed to vesicle preparations containing the antigen as the only ganglioside. In contrast, vesicle preparations containing mixtures of gangliosides failed to reduce specific antigen binding in ELISA.

4. Discussion

In the present study it was tested whether antibodies directed against major gangliosides of nerve and tumour cells [15,16,23] are able to detect their respective antigens in ganglioside mixtures. Antibody binding was strongly reduced by the presence of other gangliosides. This loss of antibody sensitivity occurred not only in ELISA, i.e. after ganglioside binding to a plastic surface, but also if gangliosides were absorbed to silica gel TLC plates or incorporated into lipid vesicles.

Quantitation of ganglioside was considered applying a method of standard addition to ELISA with crude ganglioside

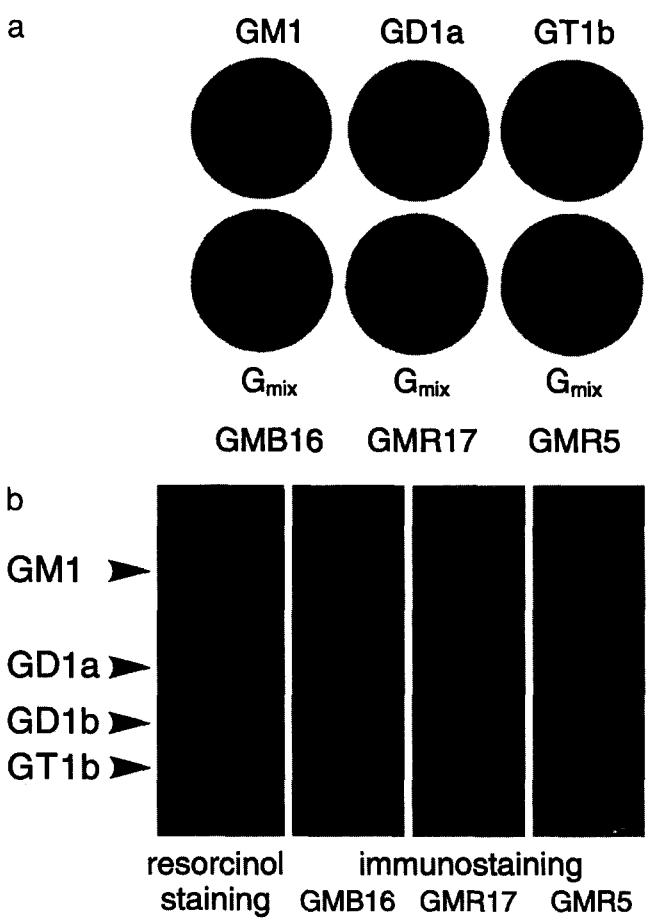

Fig. 4. Immunostaining of G_{mix} or pure standard gangliosides dotted on TLC plates without separation (a) compared to resorcinol staining and immunostaining of G_{mix} separated by TLC (b). (a) 2 nmol of pure standard ganglioside or G_{mix} containing 2 nmol of the respective antigen (i.e. 9.1, 5, and 10 nmol G_{mix} for staining with GMB16, GMR17, and GMR5, respectively) was absorbed to TLC plates and immunostained with the antibody indicated. For staining with GMB16, the site of ganglioside application had been surrounded by a dotted line, for staining with GMR17 the centre of the site of application had been marked. (b) Per lane 8 nmol G_{mix} was chromatographed and stained as indicated.

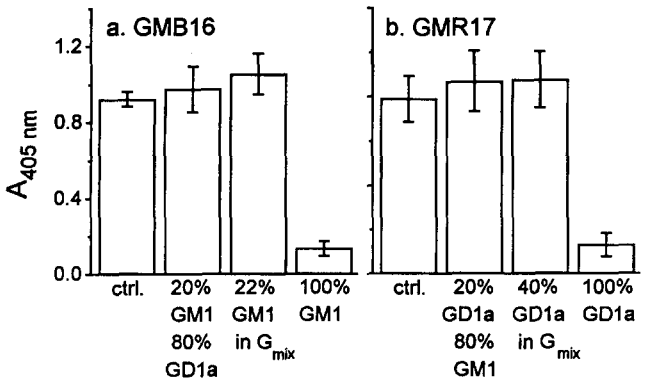

Fig. 5. Reactivity of mAb GMB16 with 100 pmol of GM1 (a) or mAb GMR17 with 100 pmol of GD1a (b) in ELISA. Prior to ELISA the antibodies were incubated with lipid vesicle preparations (1 h, RT). Per assay 20 μl of antibody solution (1:10 in 10% BSA/PBS) was added to 80 μl of vesicle preparation containing 800 pmol of ganglioside (see Section 2), ganglioside composition as indicated). Vesicle preparations without gangliosides were used as controls (ctrl.). Values are means (± S.D.) of 5 determinations each.

extracts from rat brain (Fig. 3). Since determination of individual gangliosides still requires tedious purification procedures [22], such a method appears highly desirable. However, a rise in relative antigen content caused a non-linear increase of antibody binding (see Fig. 2). Therefore, addition of antigen must lead to changes in antibody binding sensitivity. For determination of GD1a, substantial relative amounts of GD1a (12–35% of sialic acid content) had to be added. This may be the reason for the poor quality of the linear regression and could explain that compared to published values [22,30] the content of GD1a was estimated too low. In contrast, the good estimate for the content of GD3 and the highly significant linear regression indicate that the sensitivity of the antibody was only slightly affected by the small relative amount of GD3 added (1.8–6.8% of sialic acid content). In conclusion, the use of the ELISA system in quantitative detection of gangliosides seems limited. However, addition of GD3 or GD1a resulted in significant increases of antibody binding. Therefore, the ELISA appears suited to detect variations between small samples of similar composition, as for example ganglioside contents before and after a given treatment.

Although little is known about the organisation of gangliosides in cell membranes, studies with phospholipid vesicles suggest that gangliosides form enriched microdomains [31,32]. Their state of aggregation seems to influence the access of enzymes [33–35] and 'crypticity', i.e. the inaccessibility of gangliosides to ligands like enzymes and antibodies, is described in various systems. These phenomena have mostly been attributed to properties like composition of the ceramide moiety, phospholipid environment, or shielding effects by other molecules (see [36] and [37] for references). In contrast, the present study clearly demonstrates that changes in ganglioside composition alone strongly influence accessibility of gangliosides in vitro. This is consistent with reports that antibody binding to gangliosides of melanoma cells in situ depends on the ganglioside composition of the respective cell line [36,37]. Thus, it appears likely that binding properties of antiganglioside antibodies as revealed by ELISA reflect at least some of the features of antibody binding to cell surface structures. Therefore, the described properties of antiganglioside antibodies in vitro also stress the question of the structures revealed by the use of these antibodies in situ. Concerning surfaces of tissue sections or cells the antiganglioside antibodies may rather detect structures of homogeneous ganglioside composition than ganglioside antigens that occur in clusters with other ganglioside species. Besides the known features of ganglioside crypticity, this possibility should be considered, whenever gangliosides are traced by antibodies. To test how ganglioside composition influences antibody binding, the convenient ELISA procedure could prove to be a valuable tool. Thus it may contribute to a better understanding of antiganglioside antibody binding in immunohistochemistry as well as in antibody-directed tumour detection and therapy.

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